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Impact of cell-wall structure and composition on plant freezing tolerance

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Abstract

Many plants experience freezing temperatures that can be damaging and even lethal. Current climate projections suggest that freezing events are likely to increase in early autumn and late spring, at times when plants are unprepared to deal with them. Previous literature has highlighted specific mechanical properties of the plant cell wall that may impact upon freezing tolerance. For example, the limiting pore size of the cell wall can influence ice nucleation and growth, whilst cell-wall stiffness can alleviate damage from freeze-induce dehydration. More recently, there is increasing evidence that the wall undergoes major modifications in order to prepare for freezing stress, with the observation that cell-wall thickness increases and differential regulation of genes encoding cell-wall modifying enzymes occurs after exposure to cold temperatures. These findings suggest that cell-wall structure or composition are necessary and contribute to plant freezing tolerance. With the advent of molecular genetic techniques, we can now explore in further detail what aspects of the cell wall are important to prevent freezing damage, and identify targets to develop plants with enhanced freezing tolerance in the future.

1 Introduction

Changing environmental conditions can have major impacts on plant survival and crop productivity. As sessile organisms, plants are unable to avoid the variety of biotic and abiotic stresses experienced throughout their lifecycle and thus must protect themselves against damage. Unlike animal cells, plant cells are characterised by the presence of a cell wall (CW); a three-dimensional matrix surrounding plant protoplasts (McNeil *et al.*, 1984; Carpita and Gibeaut, 1993; Cosgrove, 1993) that provides strength and integrity to the cell as well as facilitating growth, cell differentiation, intercellular communication and water movement (Cosgrove, 2005). The CW defines cell shape (Sapala *et al.*, 2018) and contributes to a range of mechanical properties of specialised cell types, providing, for example, guard cells with the ability to open and close stomatal pores (Woolfenden *et al.*, 2018). The wall is also a first line of defence against biotic stress, acting as a barrier to infecting microbes (Hamann, 2012). CW composition is highly varied between plant species as well as between different tissues (Burke *et al.*, 1974; Knox, 2008; Rancour, Marita and Hatfield, 2012), but is generally comprised of three distinct sections identified via their polysaccharide content. The middle lamella (ML) and the primary cell wall (PCW) are secreted from the cell first; the ML is a shared layer rich in pectin that facilitates cell-cell adhesion, amongst other functions (Brett and Waldron, 1996; Zamil and Geitmann, 2017), whilst the PCW is formed of a fibrous network of cellulose microfibrils embedded in a matrix of pectic and hemicellulosic polysaccharides and protein (O'Neill and York, 2003). The secondary cell wall (SCW) is not synthesised in all cells but restricted to those no longer expanding and requiring supplementary strength such as sclerenchyma, tracheids and xylem vessels (Meents, Watanabe and Samuels, 2018). Cellulose makes up approximately 60% of the SCW and is structurally different from PCW cellulose due to a higher degree of crystallinity and polymerisation, resulting in microfibrils that are stronger and more rigid (McNeil *et al.*, 1984). Approximately 30% of the SCW is lignin (Campbell and Sederoff, 1996) and the final major component is hemicelluloses, which can form up to 40% of the wall dependent on species and cell type (Scheller and Ulvskov, 2010).

The CW is a dynamic structure that undergoes constant remodelling in response to growth signals and to a variety of external stimuli that include biotic and abiotic stresses (Hamann, 2012; Malinovsky, Fangel and Willats, 2014; Le Gall *et al.*, 2015; Ezquer *et al.*, 2020). This would suggest that its structure and composition need to be customised to protect the cell effectively from whatever

condition is facing the plant at any one time. Many of these changes to CWs that occur in response to external stress are brought about by transcriptional regulation of CW genes (Tenhaken, 2015; Houston *et al.*, 2016). Several studies have highlighted an extensive role for the CW in abiotic stress responses including heat, salt, drought, cold and freezing stresses (Wu *et al.*, 2010; Zhao *et al.*, 2011; Chen *et al.*, 2018; Liu *et al.*, 2019). There is also increasing evidence that sensing changes in CW integrity elicits repair and maintenance of the wall in response to both biotic and abiotic stress damage. CW integrity mechanisms have been reviewed comprehensively elsewhere and will not be discussed here (Hamann, 2012; Voxeur and Höfte, 2016; Novakovic *et al.*, 2018; Rui and Dinneny, 2020).

In this review, we focus on the contribution of the CW to freezing tolerance (FT) and on how plants may remodel their CW in preparation for later freezing conditions. Recently, there has been growing interest in how CW composition is altered in response to the cool temperatures that precede winter and how this may protect against subsequent freezing damage. The identification of specific chemical constituents or structures within the CW that can reduce damage to the plant from freezing could provide targets for crop improvement in the future.

2 Cell-wall structure and composition

2.1 Cellulose and hemicelluloses

Cellulose represents approximately 20-30% of PCWs (McNeil *et al.*, 1984) and is formed of an unbranched chain of β-(1,4)-glucose residues (Gardner and Blackwell, 1974). Between 30 and 100 cellulose chains come together to form a microfibril, which can wind around the circumference of the cell many times. The crystalline structure formed by cellulose chains contributes considerably to CW strength and this network is strengthened further by the hemicellulosic cross-links formed between microfibrils (Park *et al.*, 2015). Hemicelluloses vary greatly in structure between species and even cell types, and can consist of xyloglucans, xylans, mannans, mixed-link glucans and arabinogalactans among others (Brett and Waldron, 1996). Xylan and arabinoxylan are the major hemicelluloses present in monocot CWs (Burke *et al.*, 1974), whereas xyloglucan (XyG) is the main hemicellulosic constituent of dicots, comprising 20-25% of the PCWs of sycamore cell cultures (McNeil *et al.*, 1984). XyG consists of a backbone of β-(1,4)-linked glucose residues to which various amounts of D-xylose, D-galactose and L-fucose are attached (Hayashi, 1989). XyG chains bridge cellulose microfibrils via

hydrogen bonds to form a strongly tethered network, which can be controlled by the substitution of different sugars to the XyG backbone (Levy, Maclachlan and Staehelin, 1997).

2.2 Pectins

Pectins are the most complex polysaccharides of the CW and are rich in galacturonic acid (GalA), rhamnose (Rha), arabinose (Ara) and galactose (Gal) sugar residues. They are found in the PCW of dicots where they also make up a large proportion of the ML, though make a lesser contribution to CWs of monocots (Burke *et al.*, 1974). CW pectins comprise the pectic domains homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II), each characterised by their sugar side-chains, as well as arabinans, galactans and arabinogalactans. Pectins form covalent links with each other, resulting in the formation of the complex structure of the CW (Jarvis, 1984; Caffall and Mohnen, 2009). Like hemicelluloses, pectins may also form interactions with cellulose, although this can depend on growth status of the CW (Wang, Zabotina and Hong, 2012; Phyo *et al.*, 2017). HG polymers of α-(1,4)-linked D-GalA residues are the most abundant CW pectin (Caffall and Mohnen, 2009). HG chains can be modified by the addition or removal of *O*-methyl and *O*-acetyl esters via the activity of pectin methyl-esterase and pectin acetyl-esterase enzymes respectively (Pelloux, Rustérucci and Mellerowicz, 2007; Philippe, Pelloux and Rayon, 2017). When pectin is secreted into the apoplast HG domains exist in a highly methyl-esterified state; subsequently pectin methyl-esterases (PMEs) can remove methyl-esters from pectins, rendering them more amenable to forming Ca^{2+} cross-links ("Ca^{2+} bridges") with other HG chains, creating so-called 'egg-box' structures (Jarvis, 1984; Willats, Orfila, *et al.*, 2001). RG-I is made up of the disaccharide repeat [-α-D-GalA-(1,2)-α-l-Rha-(1,4)-] with side chains of linear or branched α-L-arabinofuranosyl (Ara*f*) or β-D-galactopyranosyl (Gal*p*) as well as other glycosyl residues, on 20-80% of Rha residues dependent on species and cell type (Lau *et al.*, 1985; Ridley, O'Neill and Mohnen, 2001). RG-II is structurally very different to RG-I. The α-(1,4)-linked D-GalA backbone is believed to be continuous with HG chains, with each unit of RG-II formed of 7-11 GalA residues and six side chains (A-F) made up of 13 different monosaccharides (Darvill, McNeil and Albersheim, 1978; Stevenson, Darvill and Albersheim, 1988; Ndeh *et al.*, 2017). The majority of RG-II exists as a dimer, with two monomeric units joined via a borate ester cross-link between the D-apiosyl (Api) residues present in side chain A (Kobayashi, Matoh and Azuma, 1996; O'Neill *et al.*, 1996).

2.3 Cell-wall proteins

Proteins are an important component of the CW and can be structural or enzymatic. The major structural proteins found in the CW are the extensins; hydroxyproline-rich glycoproteins (HRGPs) that bind to cellulose and facilitate the 'locking' of microfibrils (Carpita and Gibeaut, 1993; Showalter, 1993). An increased amount of extensin in the CW is generally correlated with cessation of growth (Sadava, Walker and Chrispeels, 1973). Arabino-galactan proteins (AGPs) are another type of HRGP that contain arabinogalactan chains (Sommer-Knudsen, Bacic and Clarke, 1998). The CW is only able to grow after stress relaxation, which requires the activity of wall loosening enzymes such as expansins, endoglucanases, endotransglycoylases and PMEs (Cosgrove, 2005, 2016a, 2016b). The most common enzymes for wall loosening are expansins which have been shown to loosen the CW without impacting wall strength, suggesting they do not cut linkages (Yuan, 2001). Xyloglucan endotransglycosylase/hydrolase (XTH) enzymes cut xyloglucan chains and join the end onto that of another xyloglucan or to water (Cosgrove, 2016b). PMEs and their inhibitors (PMEIs) are encoded by large multigene families, which is likely to reflect the diversity of their roles in CW modification, suggesting de-methylation is a tightly controlled process (Pelloux, Rustérucci and Mellerowicz, 2007). Other enzymes such as polygalacturonase (PG) and pectin acetyl-esterase (PAE) degrade and modify pectins respectively within the CW (Cosgrove, 2016b).

3 Ice in plants

Plant species respond differently to temperatures below the freezing point of water. Tropical plants are unlikely ever to experience such temperatures and indeed often exhibit chilling injury at temperatures as high as 10-12°C (Lyons, 1973). Plants that grow in temperate regions are generally chilling-resistant but can vary in their FT.

Most of the damage that occurs to plants during and after exposure to sub-zero temperatures is due to extracellular ice formation. Ice forms initially in the larger vessels where the dilute sap has a higher freezing point than that of the more concentrated cytoplasmic contents (Asahina, 1956). Ice can then spread throughout the plant from nucleation points, at the expense of water vapour and surface film on the CW. Ice crystals can form as a result of homogeneous nucleation (where a collection water molecules form a nucleus, but are much more likely formed through heterogeneous nucleation, catalysed by ice-nucleating particles such as inorganic molecules or ice-nucleating bacteria (Lindow,

Arny and Upper, 1982; Sakai and Larcher, 1987). Ice may also enter the plant from external sources via crystals nucleated on the outer surface of leaves through stomata, hydathodes, or cracks in the cuticle surface (Wisniewski and Fuller, 1999). Ice will form mainly in the gaps between cells (intercellular spaces) as shown in Figure 1a, where there is space for crystals to grow but generally cannot propagate across the lipid plasma membrane into the symplasm. However, it has been suggested that if cooling is rapid enough, at very low temperatures, ice crystals could form that are small enough to penetrate the CW and/or plasma membrane, inducing intracellular freezing and cell death, although this is likely to require rates of cooling that are higher than any experienced in nature (Levitt, 1980). This rapid *de novo* intracellular ice formation has been observed experimentally, however, it can also occur in response to moderate cooling rates if very low temperatures are reached and always results in cell death (Weiser, 1970; George and Burke, 1976; Levitt, 1980). Larger ice crystals may damage membranes by shearing or laceration as highlighted in Figure 1b (Mazur, 1963; Levitt, 1980). <Figure 1 near here>

The formation of extracellular ice within the plant apoplast results in a secondary freeze-induced dehydration stress, with ice formation lowering the water potential of the apoplast and causing water to diffuse out of the cell down the water potential gradient (Levitt, 1980; Pearce, 2001). The majority of damage to plant tissues that has been ascribed to freezing conditions can be attributed to this dehydration event (although at very low temperatures, protein denaturation adds to the problems plants experience (Thomashow, 1999)). If the temperature continues to decrease, water will continue to diffuse to points of extracellular ice due to vapour pressure differences inside and outside of the cell, thus increasing dehydration (Gusta, Burke and Kapoor, 1975). Such dehydration results in constriction of the plasma membrane (plasmolysis, Figure 1c) or even collapse of both the plasma membrane and the cell wall (cytorrhysis), which are intimately connected (Levitt, 1980). The plasma membrane, tonoplast and thylakoid membrane become damaged when dehydration exceeds the tolerance of the cell (Steponkus *et al.*, 1977; Steponkus, 1984; Murai and Yoshida, 1998b). In addition, dehydration stress leads to a phase separation of the membrane from a bilayer to a non-bilayer structure thus disrupting compartmentalisation (Stout, Majak and Reaney, 1980; Pearce and Willison, 1985). In response to freeze-induced dehydration, parts of the plasma membrane are removed as endocytotic vesicles, allowing a reduction in membrane surface area to accommodate the reduced volume of protoplasm (Dowgert and Steponkus, 1984). Upon thawing, osmotic expansion

upon re-entry of water to the protoplasm causes plasma membrane rupture as these vesicles cannot be quickly reincorporated. This results in a form of injury known as ‘expansion-induced lysis’ (EIL) (Uemura *et al.*, 2006).

4 Plant freezing tolerance

4.1 Supercooling and freezing point depression

Plants that can survive freezing temperatures either do so by tolerating ice formation in their tissues or by freeze avoidance mechanisms, which comprise the use of antifreeze proteins, ice barriers and supercooling of water (Gusta and Wisniewski, 2013; Baxter, 2014). Supercooling refers to the “depression of the freezing temperature of a liquid below its equilibrium freezing point” and constitutes the main survival mechanism in some species facing sub-zero conditions (Reyes-Diaz *et al.*, 2006). Some woody plants are able to ‘deep supercool’ to as low as -40°C in the winter, avoiding the formation of ice in some tissues even at the most severe temperatures (Burke *et al.*, 1976).

Supercooling is distinct from freezing point depression; freezing point depression occurs due to the presence of solutes within cellular and extracellular fluids, reducing the freezing point relative to the pure solvent (0°C for water). For dilute solutions, there is a well-known relationship between the freezing point depression and the concentration of a solute known as colligative freezing point depression (see for example (Atkins and De Paula, 2010)). In measurements in *Canola* leaves that had acclimated to freezing conditions, the total aqueous concentration of sugars was less than 0.4 mol Kg^{-1}. The colligative freezing point depression can be computed to be approximately 0.7°C. For temperatures below -0.7°C, the liquid state can only be preserved by supercooling. Freezing of the cell sap in these experiments was only observed to occur at temperatures less than -10°C, indicating that the solutions were indeed able to substantially supercool (Gusta *et al.*, 2004).

Supercooling is also associated with plant structure; for example, small cell size and a lack of intercellular spaces, which may partly be a consequence of CW properties (Asahina, 1956; Pearce, 2001). Supercooling is less favoured in wide-diameter spaces like the xylem and cannot occur in the presence of nucleating materials (Tyree and Dixon, 1986; Zhang *et al.*, 2016). Ice barriers are physical structures in plants that prevent ice crystals from contacting water molecules in adjacent tissues and initiating their freezing. Such physical barriers play an important part in allowing some tissues to supercool preferentially, ensuring their survival. Some organs are thus prevented from

freezing, such as hardy overwintering vegetative buds (Kuprian *et al.*, 2014; Wisniewski, Gusta and Neuner, 2014; Neuner *et al.*, 2019).

4.2 Cold acclimation

Many temperate plants can increase their FT through a process known as cold acclimation (CA). Levels of FT increase after exposure to low non-freezing temperatures in the range 0°C to 5°C, as typically experienced during autumn by temperate plants before winter frosts occur (Thomashow, 1999). This process, also known as “cold-hardening” has been studied for many decades (Levitt, 1980) and involves a vast array of transcriptional, biochemical and physiological changes that together make the plant more resilient to freezing (Hannah, Heyer and Hincha, 2005; Kaplan *et al.*, 2007). A large body of research into the molecular basis of CA has been carried out using the genetic model plant *Arabidopsis thaliana* (Warren *et al.*, 1996; Thomashow, 2010). The current knowledge on the process of CA has been fully reviewed in a number of articles (Xin and Browse, 2000; Chinnusamy, Zhu and Zhu, 2007; Knight and Knight, 2012).

A key part of CA is to stabilise membranes and membrane proteins through alterations to lipid composition (Yoshida and Uemura, 1990; Uemura, Joseph and Steponkus, 1995; Kawamura and Uemura, 2003; Uemura *et al.*, 2006). CA reduces the occurrence of EIL by avoiding the largely irreversible loss of plasma membrane surface area associated with the production of endocytotic vesicles (see section above) and instead conserving membrane surface area through formation of exocytotic extrusions, believed to be associated with a higher proportion of phosphatidylcholine (Gordon-Kamm and Steponkus, 1984b, 1984a). The accumulation of sucrose and other compatible solutes not only acts to retain water in the protoplasm, thus reducing dehydration but can also protect membranes during freezing stress, possibly by binding to the membrane or affecting adjacent water structure, ultimately preventing membrane fusion and subsequent injury (Rudolph and Crowe, 1985; Strauss and Hauser, 1986). Molecular chaperones have been shown to interact with proteins in order to prevent denaturation (Guy, Haskell and Li, 1998).

Specific proteins within the plant can inhibit ice nucleation or growth. Ice-binding proteins (IBPs, also known as anti-freeze proteins (AFPs)), adsorb to ice crystals and prevent growth of ice-nuclei, as well as preventing ice nucleation by bacteria (Kaku, 1973; Griffith *et al.*, 2005; Bredow and Walker, 2017).

Plants such as Arabidopsis limit ice nucleation to prevent damage from freezing by producing IBPs that decrease nucleation temperature in the apoplast (Bar Dolev, Braslavsky and Davies, 2016).

Transcriptional reprogramming during CA is extensive, the number of genes differentially expressed under cold exposure estimated to be in the region of 9-10,000 and including both up- and down-regulated genes (Hannah, Heyer and Hincha, 2005; Calixto *et al.*, 2018). The best studied part of the transcriptional response to CA is that brought about by the action of the CBF (C-repeat binding factor) transcription factors (Shi, Ding and Yang, 2018). In Arabidopsis, three closely related family members, CBF1, 2 and 3, also known as DREB1B, 1C and 1A respectively regulate the expression of many *COR* (cold-regulated) genes that encode proteins with roles in CA (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Shinwari *et al.*, 1998). The CBFs are conserved across freezing tolerant monocot and dicot species (Jaglo *et al.*, 2001). Whilst the CBF transcription factors control many of the most cold-responsive genes, they regulate only approximately 480 gene targets (Zhao *et al.*, 2016), meaning that CBF-independent transcriptional events must also contribute to CA (Vogel *et al.*, 2005).

5 Freezing tolerance and the cell wall

Several decades ago, a number of studies aimed to prove whether the CW plays a positive role in FT; however, some initial results were confusing, particularly those from studies using protoplasts. Several studies reported no difference between the FT of intact tissues and isolated protoplasts (Siminovitch, 1979; Singh, 1979). Furthermore, suggestions that the CW was detrimental during freezing events arose from studies in which protoplasts were observed to have greater FT than intact cells (Tao, Li and Carter, 1983; Murai and Yoshida, 1998a). Despite these findings, early studies suggested the CW acts as a barrier to ice nucleation (Ashworth and Abeles, 1984) and growth of ice (Yamada *et al.*, 2002), allowing supercooling of water and thus was likely to contribute positively to FT. CW differences observed between hardy and non-hardy varieties of crop species suggest certain CW properties can confer advantages during freezing events. Ultrastructural differences were observed between the CWs of different *Solanum* species with a frost-resistant cultivar having a thicker wall than the frost-susceptible one (Chen, Li and Cunningham, 1977). In Arabidopsis, comparison of different accessions showed that plants that accumulated more CW material during CA were more freezing tolerant than those that accumulated less (Takahashi *et al.*, 2019).

5.1 Cell-wall tension in dehydration stress and cavitation

During freezing conditions and the formation of extracellular ice, the reduced vapour pressure of ice relative to the aqueous cell contents establishes a water potential gradient, resulting in cell dehydration and ultimately collapse, illustrated in Figure 1c. However, it has been hypothesised that CWs of increased stiffness allow plants to reduce the severity of freeze-induced dehydration (Rajashekar and Lafta, 1996), cell collapse (Pearce, 1988) and CW deformation (Fujikawa, Jitsuyama and Kuroda, 1999). As water osmoses out of the cell, a stiff CW will resist the volume change, placing the CW and liquid contents under tensile stress (i.e. the liquid inside the cell is at a lower pressure compared to outside the cell, often refered to as 'negative pressure') (Hansen and Beck, 1988; Zhu, Steudle and Beck, 1989; Zhu and Beck, 1991; Vincent *et al.*, 2014). There are thus two competing potentials; the presence of extracellular ice favours water movement out of the cell, but the lower pressure inside the cell favours water movement into the cell. A dynamic equilibrium is therefore established between a concentrated (yet still liquid) cell interior, and extracellular ice. A CW of low stiffness would have little resistance to deformation upon water moving out of the cell, so could only establish modest tensile stresses and negative pressures. This means there would be little resistance to water moving out of the cell to form extracellular ice, resulting in severe cellular dehydration. Thus, in cells with stiffer CWs, extreme dehydration and desiccation can be negated. In freezing events, the reported negative pressures generated inside cells lie between appromimately -1 MPa and -10 MPa (Rajashekar and Lafta, 1996; Cochard, 2006), depending on plant species, tissue type, and CW structure. Such large negative pressures mean that vapour bubbles can form, or dissolved gasses come out of solution, leading to cavitation or embolism (Tyree and Dixon, 1986; Tyree and Sperry, 1989). A large body of literature is devoted to the specific study of cavitation and embolism in xylem vascular tissue (with associated severe conequeces for disruption of the transpiration stream (Tyree and Dixon, 1986; Tyree and Sperry, 1989)), which has been reviewed previously (Cochard *et al.*, 2013). In non-xylem tissue however, cavitation is suggested to be lethal because the cavitation event nucleates intracellular ice (Rajashekar and Burke, 1996; Barrow *et al.*, 2012).

Interestingly, evidence suggests that CW-mediated negative pressures cannot be formed in suspension-cultured cells. Rajashekar and Lafta (1996) noted that both unhardened (non-cold-acclimated) and cold-acclimated cultured cells had similar freezing properties and little resistance to collapse, in contrast to intact tissues where cold-acclimated leaves resisted intracellular freezing down

to lower temperatures than unhardened leaves. It was therefore suggested that tissue organisation is responsible for the ability of cells to resist deformation (a key feature of cellular tissue mechanics (Gibson, 2005)), enabling negative pressure formation and the consequent dehydration resistance.

From the perspectives of preventing dehydration and cavitation, we may expect a trade-off in CW stiffness: too low a stiffness results in severe dehydration stress or cell collapse, whereas too high a stiffness results in the formation of large negative pressures and cell death through cavitation. This however remains to be investigated, and a recent study suggests that the trade-off may impact the plant not just on a cellular level, but on a whole-organ level, whereby the xylem preferentially undergoes cavitation to prevent ice propagation throughout the leaf (Arias *et al.*, 2017).

5.2 Cell-wall structure in the prevention of ice propagation

Through computing the free energy change of ice entering a narrow pore, it has been shown that the freezing point of water in confined geometries should be lower than that of bulk water; see for example (Mazur, 1965; Homshaw, 1980). Experiments using glass particles of well-defined pore size validate this theory, showing that for example, ice formation in pores of diameter of 4 nm can occur only at temperatures between -15°C and -25°C (Ashworth and Abeles, 1984). In plants, the nm-scale pore size of the CW is therefore recognised as playing a key role in preventing ice propagation from extracellular sources into the cell (Wisniewski, Ashworth and Schaffer, 1987; Rajashekar and Lafta, 1996), illustrated in Figure 1b.

Pectin plays a key role in determining wall porosity and thus has the potential to influence the fate of water in plants at low temperatures. Treating CWs of *Glycine max* roots with a pectinase increased pore size, whereas treatment with protease or cellulysin had no effect (Baron-Epel, Gharyal and Schindler, 1988). Treatment of stem sections of *Prunus persica* and *Cornus florida* with pectinase to cause near complete digestion of the pit membrane resulted in a reduced ability of the xylem to deep supercool (Wisniewski, Davis and Schaffer, 1991). The CW in the pit membrane of xylem ray parenchyma, cells that neighbour xylem tissue, was shown to determine the propensity for deep supercooling, which relies on the porosity and permeability of the pit membrane (Wisniewski, Ashworth and Schaffer, 1987). It was later shown that this porosity is related to pectin structure within the xylem tissues which form a barrier resistant to water loss and growth of ice (Wisniewski and Davis, 1995).

It is important to note, however, that reducing CW porosity may have a dual function in both preventing ice entering the cell, and improving the mechanical strength required to maintain negative pressures and supercooled cell contents. It was shown that when *C. album* cells were cultured in boron-deficient medium to reduce the formation of borate diester linkages between RG-II pectin chains, CW rupture occurred more easily than those supplemented with boric acid, suggesting that smaller pores result in a stronger CW (Fleischer, Titel and Ehwald, 1998). Further research will be required to distinguish between the direct effect of CW porosity and strength on FT. Although CW tensile strength (the maximum tension the CW can withstand before breaking) has been postulated to enable cells to resist mechanical strain imposed by the presence of large extracellular ice masses (Smallwood and Bowles, 2002), further studies are necessary to evaluate the magnitude and severity of the consequences of this effect.

6 Cell-wall modifications during cold acclimation

As well as observing CW differences between hardy and non-hardy plant species under normal growth conditions, research has shown that the CW is actively remodelled during CA. Many of these modifications impart mechanics that have been described as important for FT. Table 1 highlights the modifications that have been observed in the CWs of varying plant species after a period of CA. At a gross level, CW thickness has been found to increase in cells of several different species during CA (Huner *et al.*, 1981; Griffith and Brown, 1982; Griffith *et al.*, 1985; Stefanowska *et al.*, 1999; Tanino *et al.*, 2013), as has overall CW content (Weiser, Wallner and Waddell, 1990; Kubacka-Zebalska and Kacperska, 1999; Solecka, Zebrowski and Kacperska, 2008; Takahashi *et al.*, 2019), both of which may contribute to CW strength and thus decrease injury during freezing. It has been suggested that a thicker CW may also facilitate supercooling (Sakai and Larcher, 1987), perhaps due to the relationship with CW pore size, as increased cross-linking (thus decreased pore size) in the CW was shown to correlate with increased thickness (Ishii, Matsunaga and Hayashi, 2001). Changes in the mechanical properties of the cell have also been reported, with elastic modulus, i.e. the stiffness of the CW, shown to increase after CA (Rajashekar and Lafta, 1996; Solecka, Zebrowski and Kacperska, 2008; Scholz *et al.*, 2012; Arias *et al.*, 2015). Scholz *et al.* (2012) showed that cold desert shrub species with a higher elastic modulus experienced less injury when exposed to -20°C. These studies have led to the idea that CW stiffness and/or CW strength may be associated with resilience to freezing conditions. In future studies where CW strength (maximum tensile stress before breaking)

and CW stiffness (elastic modulus) contributions to FT are measured, these two properties must be properly distinguished. <Table 1.1 near here>

6.1 Cell-wall transcriptome

Analysis of the cold-induced transcriptome has highlighted the differential regulation of CW related genes during CA and sub-zero acclimation (SZA) in several plant species (Kreps *et al.*, 2002; Seki *et al.*, 2002; Hannah, Heyer and Hincha, 2005; Herman *et al.*, 2006; Lucau-Danila *et al.*, 2012; Zhao *et al.*, 2012; Le, Pagter and Hincha, 2015; Tenhaken, 2015; Takahashi *et al.*, 2019) suggesting the CW is highly modified during CA. The overrepresentation of the CW group in down-regulated genes observed by Hannah et al. (2005) likely reflects the retardation of plant growth observed with cold exposure. However, further studies have shown that differential gene expression is not solely for the purpose of ceasing CW growth. In *Triticum aestivum* for example, hemicellulose and pectin synthesis were found to decrease with initial cold exposure, but recovered or even increased after 24 hours of cold (Zabotin *et al.*, 1998).

In Arabidopsis, some of the differentially expressed CW-related genes are part of the *CBF*-controlled regulon with 15 CW-modifying genes found to be induced by cold and down-regulated in *cbf* triple mutants (Zhao *et al.*, 2016). Members of the *EXPANSIN* and *XTH* gene families were differentially expressed after a 24 h cold exposure, with genes being both up- and down-regulated (Kilian *et al.*, 2007; Tenhaken, 2015). As well as gene-specific differences in regulation on response to cold treatment, a difference was observed between root and shoot samples, highlighting different transcriptional responses and perhaps different FT mechanisms involving the CW in these tissues (Kilian *et al.*, 2007; Tenhaken, 2015). A study in *Pisum sativum* showed that plants differing in their frost-tolerance had varying responses to cold exposure; certain CW-remodelling enzymes were found to be up-regulated only in the frost-tolerant cultivar, suggesting that modifications of the CW are required to tolerate freezing (Lucau-Danila *et al.*, 2012). The expression of *EXTENSIN* was also shown to increase after CA in *P. sativum* (Weiser, Wallner and Waddell, 1990).

Differences were observed when comparing CA at low positive temperatures and further acclimation at sub-zero temperatures (SZA). Studies revealed that changes in the levels of CW-related gene transcripts and their encoded proteins differed between the two treatments (Herman *et al.*, 2006; Takahashi *et al.*, 2019). Takahashi et al. (2019) observed an increase in total CW content after CA as

well as differences in CW composition compared to non-acclimated samples. Although the SZA samples showed the same result for these CW properties, analysis of the extracellular proteome highlighted differences in CW proteins such as enzymes with CW-modification activity between CA and SZA samples (Takahashi *et al.*, 2019). Le et al. (2015) also showed that CW-biosynthesis genes were up-regulated during SZA in Arabidopsis. These findings suggest that the plant may continually regulate CW modification and consequently CW properties, even when temperatures fall below the normal freezing point of water in order to prevent freezing injury.

6.2 Wall-membrane attachments

In *Brassica napus* transcript levels of a putative transmembrane proline-rich protein with a predicted role in wall-membrane attachments was found to increase after 6 h at low temperature (Goodwin, Pallas and Jenkins, 1996). In Arabidopsis, levels of glycosylphosphatidylinositol (GPI)-anchored proteins varied in response to cold exposure but were generally found to increase (Takahashi, Kawamura and Uemura, 2016). GPI-anchored proteins have several roles within the cell; for example two of the proteins identified as cold-inducible have roles in cellulose deposition and pectin matrix formation (Hayashi *et al.*, 2008). GPI-anchored proteins are also regarded as promising candidates for providing wall-membrane attachments with possible roles in CWI mechanisms (Liu, Persson and Sánchez-Rodríguez, 2015). Interaction between the plasma membrane and the CW has previously been suggested to be important for mediating plant FT. It has been suggested that a mechanical stress imposed by the CW on the plasma membrane may result in membrane injury during freezing events (Murai and Yoshida, 1998a), which may explain the observed increased damage from freezing in whole cells compared to plant protoplasts (Tao, Li and Carter, 1983). It may be that wall-membrane attachments are regulated during CA to prevent possible damage that may occur from tight attachments.

6.3 Pectins

Many of the studies that have reported CW modifications during CA have shown that pectic polysaccharides in particular are subject to alterations. The pectin content of the CW was shown to increase after CA in *Brassica napus* (Kubacka-Zebalska and Kacperska, 1999; Solecka, Zebrowski and Kacperska, 2008). The activity of pectin-modifying enzymes such as PAE and PG was altered after CA in *P. sativum.* Several studies have also shown differential transcription of *PME* genes and the activity of PMEs and PMEIs (Thonar, Liners and Van Cutsem, 2006; Solecka, Zebrowski and

Kacperska, 2008; Qu *et al.*, 2011; Baldwin *et al.*, 2014). In most studies, PME activity or transcript expression was shown to increase; during sub-zero acclimation (SZA), the abundance of PMEs generally tended to increase, with a concomitant decrease in PMEIs in the CWs of *A. thaliana* (Takahashi *et al.*, 2019), and transcript expression of *PME41* increased in both roots and leaves of *A. thaliana* after a 24 h cold treatment (Qu *et al.*, 2011). PME activity also increased in *B. napus* (Solecka, Zebrowski and Kacperska, 2008) as well as in *P. sativum* (Baldwin *et al.*, 2014). PMEs regulate the degree of methyl-esterification, which is correlated with the extent of pectin cross-linking; a lower level of methyl-esterification allows pectin chains to form Ca^{2+}-bridges (Willats, Orfila, *et al.*, 2001). Indeed, a decrease in methyl-esterification was observed in *B. napus* concomitant with the increase in PME activity (Solecka, Zebrowski and Kacperska, 2008). Using antibodies that distinguish between pectins with varying levels of methyl-esterification, a lower level of methyl-esterified pectin was observed in the pit membrane of xylem parenchyma of *Prunus persica* sampled in December compared to those sampled in summer months (Wisniewski and Davis, 1995). However, several studies report opposite effects, with a decrease in PME activity observed in *Cichorium intybus* roots with decreased temperature (Thonar, Liners and Van Cutsem, 2006). Similarly, in *A. thaliana*, cold treatment resulted in increased expression of the PME inhibitor gene *PMEI13*, thus reducing PME activity (Chen *et al.*, 2018) and in *P. sativum*, the degree of methyl-esterification had increased after 20 days of cold exposure (Baldwin *et al.*, 2014). These findings suggest that the degree of pectin methyl-esterification is an important structural property for FT, but that the response to cold may be varied in different plant species and tissues. This suggestion is supported by a study that has addressed this issue directly. In wheat crowns, levels of many different CW modifying enzymes responded differentially to cold exposure in the shoot apical meristem and the vascular transition zone. It was shown that these differences in enzyme abundance resulted in the enhanced methyl-esterification status of pectins in the vascular transition zone, but not the shoot apical meristem (Willick *et al.*, 2018). This could suggest the use of different survival mechanisms utilising the CW in different tissues within one plant. This is especially likely given the different CW composition and structures observed between cell types, and could be linked to differences of CW function between tissues and organs.

The degree of pectin cross-linking has been linked to the structural characteristic of CW pore size, which as previously described has been linked to the reduction of ice nucleation and growth in the

CW. A direct measurement of limiting CW pore size showed that exposure to 4°C for 3 to 5 weeks decreased the CW pore size in cultured cells of grape stems from 3.5 to 2.2 nm, with similar reduction seen in cultured apple fruit cells. Interestingly, the proportion of acclimated cells in which intracellular ice could be detected after a freezing event was shown to be 4.3%, compared to 37.6% for non-acclimated cells (Rajashekar and Lafta, 1996). Although intracellular ice is fairly uncommon *in vivo*, this shift highlights the potential for CW structure to influence ice propagation into the cell. In these experiments, a reduction in intracellular ice was correlated with an increase in CW strength, resulting in an increase in the pressure required to rupture cells (Rajashekar and Lafta, 1996). An increase in pectin content has also been linked with an increase in CW stiffness observed in *B. napus* leaves after CA (Solecka, Zebrowski and Kacperska, 2008). There are several lines of evidence for the correlation between pectin content/structure and CW stiffness (Jones *et al.*, 2003; Moore, Farrant and Driouich, 2008; Amsbury *et al.*, 2016). For the reasons discussed earlier, these may influence FT but further direct testing of this relationship is required.

6.4 Secondary cell wall

Analysis has also shown that transcripts of genes associated with lignin biosynthesis increase in leaf tissues of *Hordeum vulgare*, *A. thaliana* and *Rhododendron catawbiense* after CA (Wei *et al.*, 2006; Janská *et al.*, 2011; Ji *et al.*, 2015), however, no change was observed in crown tissue of *H. vulgare* (Janská *et al.*, 2011). These findings correlate with the increase in activity of lignin biosynthesis enzymes phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol dehydrogenase (CAD) observed in *G. max* and frost-tolerant *Miscanthus spp.* (Janas *et al.*, 2000; Domon *et al.*, 2013), as well as an increase in phenolic compounds such as ferulic and *p*-coumaric acids (Janas *et al.*, 2000; Olenichenko and Zagoskina, 2005; Domon *et al.*, 2013). Interestingly, although increases in PAL and CAD activity were also observed in a frost-sensitive species, they were not as high as the frost-tolerant species (Domon *et al.*, 2013). In studies where lignin content of leaves was measured after CA, no change was observed (Olenichenko and Zagoskina, 2005; Ji *et al.*, 2015), although an increase was observed within crown tissues of *T. aestivum* (Olenichenko and Zagoskina, 2005). Glucoronoarabinoxylans (GAX) were also found to increase in the vascular transition zone of *T. aestivum* but not the shoot apical meristem, and in a frost-tolerant variety of *Miscanthus sp.* (Domon *et al.*, 2013; Willick *et al.*, 2018). GAX are major wall polymers that link cellulose microfibrils in

monocots, but can also cross-link to lignin by compounds such as ferulic acid to enhance wall rigidity (Carpita and Gibeaut, 1993; Hatfield, Rancour and Marita, 2017).

7 Using Arabidopsis genetic resources to demonstrate cell-wall contributions to freezing tolerance

We have described above how a number of CW modifications and characteristics have been implicated in FT either because they are associated with CA or as their presence correlates with more freezing-tolerant varieties/species of plant. Although the mechanisms via which wall components may prevent freezing damage have been hypothesised, there is still no clear answer as to how the CW protects the plant against freezing stress. It is highly likely that different species and even different tissues employ specific mechanisms to prevent freezing injury, which would explain the variety of CW modifications during cold exposure. With the advent of molecular genetic tools, it is possible to get closer to understanding the functional significance of some of these features.

7.1 Pectin cross-linking

In addition to Ca^{2+} bridges that form between HG chains, pectic cross-linking also occurs through the dimerisation of RG-II domains via borate-diester linkages (Kobayashi, Matoh and Azuma, 1996; O'Neill *et al.*, 1996). In a similar manner, this form of pectic cross-linking can contribute to the determination of CW pore size. Cultured cells of *Chenopodium album* grown in a boron-deficient medium had larger pores than those supplemented with boric acid, the mean size limit decreasing from 5.62 to 3.41 nm in growing cells with 100 μM boric acid (Fleischer, Titel and Ehwald, 1998). Cells with larger pore sizes were shown to contain only monomeric RG-II domains, whilst supplementing with boric acid resulted in an increase in the presence of dimerised RG-II associated with the decreased CW pore size (Fleischer, O'Neill and Ehwald, 1999).

Alterations to CW strength also appear to be linked to RG-II dimerisation; tensile strength and tensile modulus were decreased in hypocotyls of Arabidopsis *mur1* mutants, which exhibit reduced RG-II dimerization (Ryden *et al.*, 2003). The *MUR1* gene encodes an enzyme necessary for the synthesis of fucose, an important component of RG-II domains. In *mur1* mutants, which have severely reduced levels of CW fucose, RG-II dimerization in the CW was reduced to 50%, compared to 95% dimerization in WT (O'Neill *et al.*, 2001). One of the first descriptions of *mur1-1* tissues was an increase in brittleness (Reiter, Chapple and Somerville, 1993), referring to the break point occurring

after only small plastic deformations. Boron deficiency in roots of squash and bean was shown to reduce the CW elastic modulus (Findeklee and Goldbach, 1996; Findeklee, Wimmer and Goldbach, 1997), and hypocotyls of boron-deficient squash were shown to have more brittle and rigid tissues (Hu and Brown, 1994).

Recently, the *mur1* mutant was shown to be freezing sensitive (Panter *et al.*, 2019). A forward genetic screen for Arabidopsis mutants identified a number of *sensitive-to-freezing* (*sfr*) mutants (Warren *et al.*, 1996). The *sfr8* mutation was mapped to the *MUR1* gene suggesting a relationship between the decrease in RG-II dimerization and the increased freezing sensitivity of *sfr8*/*mur1* mutants. Further evidence for this is highlighted in the finding that supplementing *sfr8* plants with boric acid during growth, which has been shown to restore RG-dimerisation in plants (O'Neill *et al.*, 2001) was able to restore almost wild-type tolerance to freezing (Panter *et al.*, 2019). A decrease in FT was also observed in Arabidopsis *bor* mutants, which are unable to transport boron and also display a decrease in CW RG-II cross-linking (Panter *et al.*, 2019). These findings suggest that RG-II cross-linking, and possibly pectin cross-linking in general in the CW is beneficial for FT.

In support of this hypothesis, overexpression of *PMEI13* in Arabidopsis led to decreased PME activity and an increase in the degree of pectin methyl-esterification, resulting in plants that were more sensitive to freezing (Chen *et al.*, 2018). This provides evidence that the degree of methyl-esterification controlled by the action of PMEs and PMEIs is an important property for defining tolerance to freezing. Indeed, the observed increased PME activity, and a decrease in the degree of methyl-esterification after CA in several plant species would agree with this. A lower degree of methyl-esterification is linked to a higher degree of Ca^{2+} cross-linking, which like RG-II cross-linking, results in an increased stiffness and decreased CW pore size (Fleischer, O'Neill and Ehwald, 1999; Willats, Orfila, *et al.*, 2001; Ryden *et al.*, 2003), either or both of which might enhance FT. However, some of the CW related pectin modifications observed during CA do not fit this hypothesis as was discussed in section 1.6.3. Interestingly, Willats *et al*. (2001) also reported that it is the pattern of methyl-esterification as well as the degree of methyl-esterification on pectin chains that may influence other CW qualities such as porosity and stiffness, which may explain the differences observed. Again it is also likely that different species and tissues employ different mechanisms for withstanding freezing, supported by the finding that PMEs and PMEIs have highly specific expression patterns in different tissues (Pelloux, Rustérucci and Mellerowicz, 2007). It is possible that there is close regulation of

pectin structure during CA through the action of specific pectin modifying enzymes that create a structure with the properties necessary to withstand freezing events.

7.2 Hemicelluloses

A *XTH* gene, the transcript levels of which increase during exposure to cold, was also shown to impact upon FT. Arabidopsis *xth21* mutants had increased freezing sensitivity, whilst lines overexpressing the *XTH21* gene had increased FT compared to wild-type plants (Shi *et al.*, 2014), suggesting that hemicellulose structure is important for FT. Interestingly, *XTH21* expression was shown to peak after 12 h of cold exposure and return to almost control levels after 24 h (Shi *et al.*, 2014). This correlates with transcriptomic analysis of the *XTH* gene family, which showed no change in *XTH21* expression after 24 h cold exposure (Kilian *et al.*, 2007; Tenhaken, 2015). These results highlight the need for direct structural measurements of the CW after CA in order to analyse what components may impact upon FT.

7.3 Lignin and the secondary cell wall

Modifications to the SCW during CA also occur through regulation of lignin biosynthesis. However, although expression levels of many lignin biosynthesis genes were found to increase with CA, there is very little evidence that lignin content increases. Analysis of a cold-induced nuclear protein TCF1 (Tolerant to Chilling and Freezing 1) led to the discovery of a role in regulating lignin biosynthesis in Arabidopsis. Loss of TCF1 function resulted in a decrease in lignin content, but an increase in FT. Further data to support an inverse relationship between lignin content and FT came from the observation that a *pal1pal2* double mutant, (PALs act downstream of TCF1 in lignin biosynthesis) exhibited a 30% decrease in lignin content and an increase in FT (Ji *et al.*, 2015). Another CW Arabidopis mutant, *esk1*, was found to be constitutively FT (Xin and Browse, 1998). ESK1 is an *O*-acetyl-transferase that acetylates xylan, which contributes to SCW architecture (Urbanowicz *et al.*, 2014). Indeed *esk1* mutants were shown to have altered SCW composition and subsequent xylem malformation (Lefebvre *et al.*, 2011). This further implicates the secondary CW as having a possible detrimental role in FT. Lignin is a major determinant of CW stiffness (Özparpucu *et al.*, 2017), and as stated earlier, CW stiffness is related to the ability of the cell to withstand freeze-induced dehydration, but that this can result in the creation of negative pressures. Perhaps CW stiffness, particularly that related to the SCW and lignin formation, is tightly regulated to ensure the appropriate level of stiffness required for the freezing event experienced. Interestingly, a decrease in expression of a *PMEI* was

reported in the *esk1* mutant (Bouchabke-Coussa *et al.*, 2008), which correlated with a reduction in esterified pectins observed by Lefebvre *et al.* (2011) which may link the constitutive FT of *esk1* to increased pectin cross-linking.

8 Future prospects

A potential link between the plant CW and FT has been well-established. CW stiffness and pore size have been well-documented as providing the ability to withstand freeze-induced dehydration and allow cells to supercool. However, there are still unanswered questions: is CW stiffness of particular benefit or is strength more important?; is the mechanical weight burden of ice on the CW an issue?; is there an optimal degree of CW stiffness, beyond which tensions increase too much and the wall collapses?; are wall-membrane attachments beneficial or detrimental to the cell during freezing? As well as these general questions, it is also unclear specifically which CW polysaccharides contribute to FT. Analysing CW modifications during CA has led to the suggestion that pectins are an essential CW component during freezing. Pectin cross-linking in particular has been linked to CW thickness (Ishii, Matsunaga and Hayashi, 2001), strength, stiffness (Ryden *et al.*, 2003) and pore size (Fleischer, O'Neill and Ehwald, 1999), all of which have been associated with FT and CA. Indeed recent work has shown that cross-linking via RG-II dimerization and formation of Ca^{2+} bridges after pectin de-esterification appear to be beneficial for FT (Chen *et al.*, 2018; Panter *et al.*, 2019). However, *mur1* mutants also display alterations to lignification of xylem tissues, which may impact on FT (Voxeur *et al.*, 2017). The role of lignin in the contribution of the CW to FT is yet to be fully understood in light of the conflicting results obtained thus far. Determining whether lignin is beneficial or detrimental to the plant during freezing events would inform on the necessity of CW stiffness.

The degree of pectin methyl-esterification within the CW may also influence more than just Ca^{2+} cross-linking, as the pattern of methyl-esterification also appears to affect wall porosity and stiffness, as well as water holding capacity of pectins, a trait whose contribution to FT has not been fully explored (Willats, Orfila, *et al.*, 2001; Levesque-Tremblay *et al.*, 2015). Considering the observation of transcriptional modification of PMEs and PMEIs during cold acclimation, it is possible that these large gene families, encoding enzymes that likely have very specific functions, are differentially regulated to modify the CW in a distinct way to increase FT. This would be an interesting line of enquiry, as pectins appear to be important for FT even in monocots where pectins constitute only a very small

percentage of the CW (Willick *et al.*, 2018). A consideration that must be maintained is the influence of CW integrity sensing mechanisms; CW mutations that result in alterations to structure may well trigger modifications in completely separate areas of the CW. Thus, it will be necessary to ensure CW structure is completely understood before inferring specific CW components have a role in FT.

We now have the opportunity to return to a wealth of information generated by excellent plant physiologists, and advance this knowledge with the molecular genetic resources currently available to us. The contribution of specific CW polysaccharides can be assessed using plant CW mutants, and CW structure and composition can be measured using techniques such as antibody labelling (Willats, McCartney, *et al.*, 2001), radiolabelling of CW sugars (Thompson and Fry, 2001) and size exclusion chromatography (O'Neill *et al.*, 1996). It is now possible to assess these traits at a cell-specific level in a variety of different species, pertinent, given the fact that CW composition and modifications during CA are highly species- and tissue-specific. Further research into this area could provide us with a wealth of data to inform target identification for increasing FT in many different crop species.

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Figure 1

Illustration of two major functions of the cell wall (CW) in freezing tolerance. (a) Plant cell tissue showing the formation of extracellular ice crystals (blue) in the intercellular spaces. The CW is shown in brown, and the cell contents in yellow. (b) Magnifications of the CW showing ice interacting with the apoplast. For large pore sizes, ice can propagate through the CW (direction of propagation indicated

with a red arrow) and nucleate intracellular ice. This is prevented in CWs with small pore sizes. (c) In the presence of extracellular ice, water is able to diffuse out of the cell. Flexible CWs offer minimal resistance to this process, indicated by red arrows, resulting in severe dehydration and even plasmolysis. Stiff CWs enable a viable equilibrium to be established between a concentrated cytoplasm under tension and extracellular ice, indicated by the black arrow.

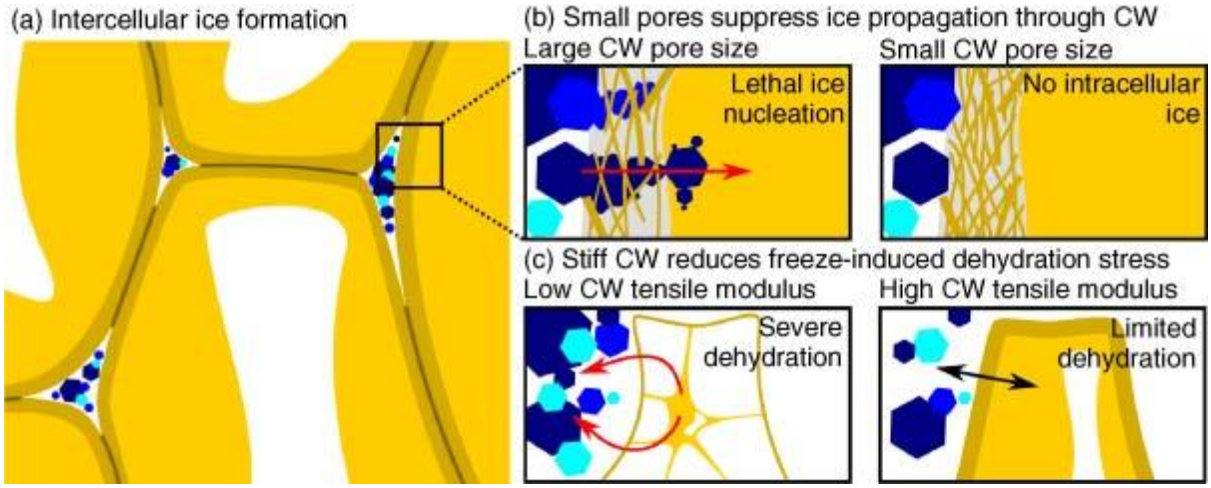

Table 1 Cell-wall modifications after cold acclimation

Species	Tissue	Acclimation	Target	Modification	Reference
Secale cereale	Leaves	4/2°C D/N, 90 d	CW thickness	Increased	Huner *et al.* (1981)
Secale cereale	Leaves	5/2°C D/N, 49 d	CW thickness	Increased	Griffith and Brown (1982)
Secale cereale	Leaves	5°C, 41, 56 and 76 d	CW thickness	Increased	Griffith *et al.* (1985)
Pisum sativum	Epicotyl	2°C up to approx. 30 d	*Extensin* transcript expression	Increased	Weiser, Wallner and Waddell (1990)
			Arabinose and hydroxyproline	Increased	
			CW content (mg g^{-1} fresh weight)	Increased after 20 d	
Prunus persica	Xylem	*in natura*	Degree of methyl-esterification	Decreased	Wisniewski and Davis (1995)
Brassica napus	Leaves	4°C, 8 h	*Proline-rich CW protein* transcript expression	Increased	Goodwin, Pallas and Jenkins (1996)
Vitis spp. and *Malus domestica*	Cell cultures	4°C, 21-35 d	CW breaking pressure (MPa)	Increased	Rajashekar and Lafta (1996)
			Limiting CW pore size	Decreased	
Broadleaf evergreen species	Leaves	3°C, 42 d	Cell tensions	Increased	Rajashekar and Lafta (1996)
Triticum aestivum	Roots	2°C, 1, 3, 6, 12 and 24 h	Hemicellulose synthesis	Initial decrease, increased after 24 h	Zabotin *et al.* (1998)

Species	Tissue	Acclimation	Target	Modification	Reference
			Pectin synthesis	Initial decrease, recovery after 24 h	
Brassica napus	Leaves	2°C, 21 d	CW thickness	Increased	Stefanowska *et al.* (1999)
Brassica napus	Leaves	2°C, 21 d	CW content (mg g^{-1} dry weight)	Increased	Kubacka-Zebalska and Kacperska (1999)
			CW Pectin (mg 100mg^{-1} CW extract)	Increased	
Glycine max	Roots	10°C, 24 h	PAL activity	Increased	Janas *et al.* (2000)
			Phenolic acids; p-hydroxybenzoic, vanillic, syringic, anisic, p-coumaric and ferulic	Increased	
			Phenolic glycosides	Decreased	
Triticum aestivum	Leaves, crown tissue	Low positive temperatures	Soluble phenolic compounds (mg g^{-1} fresh weight)	Increased	Olenichenko and Zagoskina (2005)
			PAL activity	Decreased	
			L-phenylalanine content	Increased	
			Lignin content (mg g^{-1} fresh weight)	Leaves; no change, crown; increased	
Rhododendron catawbiense	Leaves	*in natura* (Summer vs Winter samples)	*C3H* transcript expression	Increased	Wei (2006)
Cichorium intybus	Roots	*in natura*	PME activity	Decreased	Thonar, Liners and Van Cutsem (2006)
			PAE activity	No change	
Triticum aestivum	Crown tissue	3°C, 21 d then -3°C, 6 h, 1 and 3 d	*CW-related gene* transcript expression	Differential regulation	Herman *et al.* (2006)
Brassica napus	Leaves	2°C, 21 d	CW content (mg g^{-1} dry weight)	Increased	Solecka, Zebrowski and Kacperska (2008)
			CW pectin (mg g^{-1} dry weight)	Increased	
			PME activity	Increased	
			Degree of metyl-esterifcation	Decreased	
			Tensile stiffness (MPa)	Increased	
Hordeum vulgare	Leaves, crown tissue	3/2°C D/N, 1, 3, 7, and 21 d	*Lignin synthesis-related gene* transcript expression e.g. *PAL*s, *CAD*	Leaves; increased, crown; decreased/no change	Janská *et al.* (2011)
Arabidopsis thaliana	Leaves, roots	0°C, 6 h	PME activity	Increased	Qu *et al.* (2011)
			PME41 transcript expression	Increased	

Species	Tissue	Acclimation	Target	Modification	Reference
Cold desert shrub species	Shoots	*in natura* (Summer vs Winter samples)	Elastic modulus (MPa)	Increased	Scholz *et al.* (2012)
Pisum sativum	Leaves, roots	10/2°C D/N, 20 d	*CW-related gene* transcript expression	Differential regulation	Lucau-Danila *et al.* (2012)
Miscanthus spp. (FT)	Leaves	12°C, 4 and 8 d	β-D-glucan content	Increased	Domon *et al.* (2013)
			Uronic acid	Decreased	
			GAX content	Increased	
			CAD activity	Increased	
			PAL activity	Increased	
Allium fistulosum	Leaves	12/4°C D/N, 7 and 14 d	CW thickness	Increased	Tanino *et al.* (2013)
Arabidopsis thaliana	Leaves	4°C, 3, 6, 12 and 24 h	*XTH21* transcript expression	Increased; peak at 12 h	Shi *et al.* (2014)
Pisum sativum (FT cultivar)	Stipules	10/2°C D/N, 5, 10 and 20 d	Degree of methyl-esterification	Decreased after 5 d, increased after 10 and 20 d	Baldwin *et al.* (2014)
			PAE activity	Increased	
			PME activity	Decreased after 5 d, increased after 10 and 20 d	
			PG activity	Increased after 10 d, decreased after 20 d	
Arabidopsis thaliana	Leaves, roots	24 h	*XTH-gene family* transcript expression	Differential regulation	Tenhaken (2015), Kilian *et al.* (2007)
			Expansin-gene family transcript expression	Differential regulation	
Arabidopsis thaliana	Leaves	4°C, 14 days, then -3°C, 1, 2, 3 and 8 h	*CW-related gene* transcript expression	Differential regulation	Le *et al.* (2015)
Olea euroapea	Leaves	*in natura* (Summer vs Winter samples)	Elastic modulus (MPa)	Increased	Arias *et al.* (2015)
Arabidopsis thaliana	Leaves	4°C, 7 d	*BCB*, *PAL2* and *PAL4* transcript expression	Increased	Ji *et al.* (2015)
			Lignin content (mg mg^{-1} dry weight)	No change	
Arabidopsis thaliana	Leaves	2°C, 7 d	GPI-anchored proteins	Increased and decreased	Takahashi, Kawamura and Uemura (2016)
Arabidopsis thaliana	Leaves	4°C, 1, 3, 6 and 24 h	*PMEI13* transcript expression	Increased (peak at 6 h)	Chen *et al.* (2018)
Chorispora bungeana	Leaves	4°C, 1, 3, 6 and 24 h	*PMEI1* transcript expression	Increased	Chen *et al.* (2018)
Triticum aestivum	SAM, VTZ	4°C, 21 and 42 d	Apoplastic CW modifying enzymes	Mostly increased	Willick *et al.* (2018)

Species	Tissue	Acclimation	Target	Modification	Reference
			Degree of methyl-esterification	VTZ; increased, SAM; no change	
			GAX	VTZ; increased, SAM; no change	
Arabidopsis thaliana	Leaves	4°C, 7 d	Extracellular proteome CW content (mg g^{-1} dry weight)	Differential abundance Increased	Takahashi *et al.* (2019)
		4°C, 7 d then -3°C, 3 d	Extracellular proteome CW content (mg g^{-1} dry weight)	Differential abundance Increased	

BCB - blue-copper-binding protein; C3H - coumorate-3-hydroxylase; CAD - cinnamyl alcohol dehydrogenase; CW - cell wall; D/N - day/night; FT - frost-tolerant; GAX - glucuronoarabinoxylan; GPI - glycosylphosphatidylinositol; MPa – megapascals; PAE – pectin acetyl-esterase; PAL - phenylalanine ammonia-lyase; PME - pectin methyl-esterase; PMEI - pectin methyl-esterase inhibitor; PG - polygalacturonase; SAM - shoot apical meristem; TD - tillering nodes; VTZ - vascular transition zone; XTH - xyloglucan endotransglucosylases/hydrolases